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Cigarette smoking increases bronchial mucosal IL-17A expression in asthmatics, which acts in concert with environmental aeroallergens to engender neutrophilic inflammation

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Cigarette smoking increases bronchial mucosal IL-17A expression in asthmatics, which acts in concert with environmental aeroallergens to engender neutrophilic inflammation

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Abstract:

Background

Mild asthmatics who smoke cigarettes may develop unstable disease and neutrophilic infiltration of the airways, features more usually associated with severe asthmatic disease. The mechanisms giving rise to this response remain unclear.

Objective

To address the hypothesis that smoking increases bronchial mucosal production of IL-17A which acts on bronchial epithelial cells directly and in concert with other environmental stimuli to induce the production of IL-6 and neutrophil chemotaxins.

Methods

IL-17A, IL-8, IL-6, neutrophils and eosinophils was detected and quantified by immunohistochemistry in endobronchial biopsy sections from smoking and non-smoking asthmatics. Human tracheal epithelial cells (HTEpC) were cultured with IL-17A in the presence/absence of cigarette smoke extract (CSE) and aeroallergens lacking intrinsic protease activity, and IL-6 and IL-8 production measured *in vitro*.

Results

Expression of IL-17A, IL-6 and IL-8 and neutrophil numbers were significantly elevated in the bronchial mucosa of the asthmatic smokers compared to the non-smokers. Expression of IL-17A correlated with that of IL-8 and neutrophil numbers. In the smoking asthmatics, eosinophil numbers also correlated with expression of IL-8 and IL-17A. Exposure of HTEpC cells to both CSE and IL-17A increased expression of IL-6 and IL-8 in a concentration-dependent and synergistic manner.

Co-stimulation with CSE, IL-17A and aeroallergens further increased IL-6 and IL-8 production synergistically.

Conclusions

The data support the hypothesis that asthmatic smokers develop neutrophilic inflammation of the airways propagated at least partly by smoke-induced production of IL-17A which together with smoke and other environmental stimuli acts on airways epithelial cells to induce neutrophil chemotaxins.

Abbreviations:

COPD	Chronic Obstructive Pulmonary Disease
CSE	Cigarette Smoke Extract
ELISA	Enzyme-linked immunosorbent assay
FEV1	Forced expiratory volume in 1 second
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HTEpC	Human tracheal epithelial cells
IHC	Immunohistochemistry
IL	Interleukin
IQR	Interquartile range
MBP	Major Basic Protein
NHS	National Health Service
OVA	Ovalbumin
PBS	Phosphate-buffered saline
PC_{20}	Provocation concentration producing a 20% fall in forced expiratory volume in one second
REC	Research Ethics Committee
ROS	Reactive oxygen species
SEM	Standard error of the mean
Th	T helper
TLR4	Toll-like receptor 4

INTRODUCTION

Unfortunately, many asthmatics smoke cigarettes [1]. There is abundant evidence that smoking destabilises asthma [2, 3] and confers resistance to therapy [4, 5]. Smoking in asthma is accompanied by elevated inflammatory cellular infiltration of the airways, with a prominence of neutrophils [6, 7], a scenario associated with severe, difficult to control disease in non-smoking patients [8].

Respiratory epithelial cells constitute the common environmental interface for cigarette smoke, pathogens and allergens in the respiratory tract and are able to respond to the latter through pattern recognition receptors independently of the IgE responses which characterise atopy [9]. There is nevertheless a paucity of research on the possible interactions between cigarette smoke and the airways mucosal epithelium which may drive neutrophilic inflammation in mild asthma. One notable possible link in this mechanistic chain is IL-17A, which has been described to stimulate various airways structural cells to produce CXC chemokines [10-12] which are produced in excess in the airways of patients with “neutrophilic” asthma [13]. Interestingly, IL-17A production both in severe, non-smoking asthmatics [14] and in milder asthmatics who smoke [15] appears to be therapy resistant. Preliminary observations [16, 17] indicate that exposure of bronchial epithelial cells both to cigarette smoke and to IL-17A increases their spontaneous release of CXCL8 (a key chemotactic factor for neutrophils as well as “primed” eosinophils [18]) and IL-6, an “acute phase” cytokine also associated with sputum neutrophilia in asthmatics [13, 19].

Consequently we constructed the present study to address the following hypotheses: (1) Cigarette smoking stimulates the production of IL-17A, neutrophil chemotaxins

and granulocytic infiltration within the bronchial mucosa of mild, corticosteroid naïve asthmatics; (2) Cigarette smoke extract and IL-17A enhance the production of pro-neutrophilic cytokines by human airways epithelial cells both independently and synergistically; (3) This effect is further potentiated by non-antigen-specific (innate) interactions with environmental allergens. In order to address these hypotheses we quantified immunoreactive IL-17A$^+$, IL-6$^+$ and IL-8$^+$ cells as well as elastase$^+$ neutrophils and MBP$^+$ (major basic protein) eosinophils in bronchial mucosal sections from corticosteroid naïve smoking and non-smoking asthmatics. We also examined the effects of IL-17A, alone and in combination with cigarette smoke extract (CSE) and environmental aeroallergens on the production of IL-6 and IL-8 by human airways epithelial cells *ex vivo*.

SUBJECTS, MATERIALS AND METHODS

Subjects and fibreoptic bronchoscopy

In view of the school of thought that corticosteroid therapy itself may be at least partly responsible for neutrophilic infiltration of the airways in asthma [20], we elected to perform a cross sectional comparison of mild, corticosteroid naïve asthmatic smokers and non-smokers (London Bridge Research Ethics Committee, REC approval reference 06/Q0704/175; Guy's and St Thomas' NHS Foundation Trust Research and Development Department, R&D approval number RJ1 07/0069). The following definitions were used when characterising participants: (i) asthma: a history of typical symptoms for ≥ 6 months prior to screening and histamine PC_{20} ≤8 mg/ml; (ii) non-smoker: no smoking within 12 months of screening and < 0.5 pack year total history; (iii) smoker: currently smoking ≥ 5 cigarettes/week. The study was explorative and not formally powered because at the time inception of the study there were no suitable preliminary studies available to enable this.

Informed consent was obtained from all subjects who then underwent a full assessment of their medical history, physical examination, St. George's Respiratory Questionnaire, spirometry and measurement of histamine PC_{20}. Fibreoptic bronchoscopy was performed in accordance with the British Thoracic Society guidelines [21]. Up to 12 endobronchial biopsies were obtained from the subcarinae of 3rd to 7th generation bronchi.

Immunohistochemistry (IHC)

Cryostat sections (6 μm) were cut from biopsies prefixed in 4% paraformaldehyde (BDH Chemicals Ltd, Dagenham, UK), mounted on 0.1% poly-L-lysine-coated slides, dried overnight at 37°C then stored with silica gel (BDH Chemicals) at -80°C until used. Single IHC was performed as previously described [22] using the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique with monoclonal antibodies against neutrophil elastase (NE, 1:100, Clone: NP57, Dako, Ely, UK) and eosinophil major basic protein (MBP, 1:30, Clone: BMK13, Abcam, Cambridge, UK), and polyclonal antibodies against IL-6 (1:500, Abcam), IL-8 (1:500, R&D Systems, Minneapolis, MN) and IL-17A (1:200, eBioscience, Hatfield, UK). Sections were then counterstained with Mayer's haematoxylin solution (Sigma-Aldrich). Omission or substitution of the primary antibody with an irrelevant species- and isotype-matched immunoglobulin was used as a negative control. Stained cells in coded sections were counted by a single observer ignorant of their provenance in the entire area of the biopsy specimens with an Olympus BX40 microscope coupled with a Zeiss Vision KS300 imaging system (Carl Zeiss, Hamburg, Germany), which measured these areas and uniformised background pixilation automatically. Endobronchial biopsies from asthmatics have a tendency to lose some or all of their epithelium during the biopsy process and/or subsequent processing. In the present study, 6% of biopsies had a fully intact epithelium, 53% had a partially intact epithelium and 41% had no epithelium present. There was no statistical difference in the frequencies of these three outcomes in the biopsies from the non-smoking and smoking asthmatics ($p = 0.4926$). In view of this we elected to measure cell counts in the entire biopsy sections, which were expressed as the numbers of immunoreactive cells per unit area of the sections.

152 **Cigarette smoke extract (CSE) preparation**

153 Cigarette smoke extract (CSE) was prepared based on a modification of the method of
154 Carp and Janoff [23]. Two full-strength Marlboro cigarettes (filters removed;
155 Marlboro Red, Class A cigarette, Tar 10 mg, Nicotine 0.8 mg; Phillip Morris USA,
156 Richmond, VA) were combusted and the smoke bubbled through 50 ml of culture
157 medium. To accomplish this, a 50 ml syringe (Becton Dickinson, Oxford, UK) was
158 connected to a 3-way stopcock (Becton Dickinson). One of the other 2 arms of the
159 stopcock was connected to a cigarette in a holder, and the final arm was connected to
160 a glass Pasteur pipette (John Poulten, Barking, UK) submerged in a beaker containing
161 culture medium. In a fume cupboard, each cigarette was lit and cigarette smoke drawn
162 into the 50 ml syringe (to the 60 ml mark) over 10 seconds; following this the
163 cigarette smoke was immediately bubbled into the culture medium over 2 to 3
164 seconds. According to the protocol of Carp and Janoff [23], this solution was defined
165 as "100% strength". The resulting medium was then sterilised by passing through a
166 0.20 μm filter (Sartorius, Epsom, UK), diluted to the required strength and used
167 within 1 hour of preparation [23].

168 **Human Airways Epithelial Cell Cultures**

169 Human tracheal epithelial cells from mixed healthy donors (HTEpC, PromoCell,
170 Heidelberg, Germany) were cultured in accordance with the supplier's instructions in
171 bovine type I collagen coated 12 well tissue culture plates in Airway Epithelial Cell
172 Medium which contained bovine pituitary extract 0.004 ml/ml, epidermal growth
173 factor 10 ng/ml, insulin 5 μg/ml, triiodo-L-thyronine 6.7 ng/ml, holo-transferrin 10
174 μg/ml, hydrocortisone 0.5 μg/ml, epinephrine 0.5 μg/ml and retinoic acid 0.1 ng/ml

(PromoCell) and was supplemented with 1% antibiotic-antimycotic (Gibco, Paisley, UK). 24 hours prior to stimulation the culture medium was changed to a "starvation" medium (Airway Epithelial Cell Medium without hydrocortisone, epinephrine and retinoic acid supplemented with 1% antibiotic-antimycotic and 25 mM HEPES (Sigma-Aldrich)). The cells were then exposed for 24 hours to 0.1, 1.0 and 10 ng/ml of recombinant human IL-17A (PeproTech, London, UK), 0.16% CSE, 5000 SQ-U/ml *Felis domesticus* Aquagen SQ (Alk-Abello, Horsholm, Denmark) and 5000 SQ-U/ml *Phleum pratense* Aquagen SQ (Alk-Abello), then supernatants collected for analysis [24, 25]. The concentration (0.16%) of CSE employed for these experiments was determined from prior concentration/response and time course studies to stimulate maximal cytokine production in HTEpC (data not shown). After removal of culture supernatant, cellular viability was assessed under light microscopy by trypan blue exclusion. The viability of HTEpC cells when exposed to CSE under these experimental conditions remained in excess of 90% throughput the culture period.

ELISA

Concentrations of IL-6, IL-8, IL-17A and IL-17F were measured in culture supernatants using commercial ELISA (PeproTech, London, UK) in accordance with the manufacturer's instructions.

Statistical Analysis

Statistical analysis was performed using software embedded in Prism 5 for Mac OS X Version 5.0c (GraphPad Software Inc, La Jolla, CA). Data were summarised as the mean and standard error, or the median and interquartile range (IQR) as appropriate.

Data were compared following testing for deviance from a Gaussian distribution and for equality of variance using parametric analysis (parametric one-way analysis of variance, paired student's t-test, linear regression, Pearson's correlation) or non-parametric analysis (Mann-Whitney U test, Kruskall–Wallis one-way analysis of variance) as appropriate. Since the variances of the data from the asthmatic groups were not homogeneous, they were compared using non-parametric statistical analysis. Parametric statistical analysis was employed for all other comparisons since the data obtained satisfied the relevant statistical assumptions. In the IL-17A and CSE interaction experiments, synergistic interaction was defined as significant variation (using one-way analysis of variance) in the magnitude of CSE-induced cytokine expression in excess of spontaneous secretion at the concentrations of IL-17A tested. In the allergen, IL-17A and CSE interaction experiments, synergistic interaction was defined as a significant, further allergen-induced increase in the effects of IL-17A and CSE, either alone or in combination. One-way analysis of variance (ANOVA) was performed on the magnitude of the IL-17A concentration series-induced increases on cytokine secretion from baseline in the additional presence of CSE. Statistical significance was taken as $p<0.05$. P values are quoted to two significant figures. For Pearson's correlations, r is the Pearson's correlation coefficient while r^2 is the coefficient of determination.

RESULTS

Subject characteristics

Endobronchial biopsies were obtained from 10 asthmatic non-smokers and 8 asthmatic smokers (Table 1). All asthmatic smokers were current smokers with a median (IQR) pack year history of 4.7 (0.9 – 18.0) pack years, currently smoking 14.0 (6.3 – 20.0) cigarettes per day. Of those classified as asthmatic non-smokers, 2 subjects were ex-smokers. The ex-smokers had stopped smoking 3 and 14 years prior to the study, with pack year histories of 0.125 and 0.150 pack years. Asthma severity in the two asthmatic groups was equivalent, as defined by % predicted FEV_1 ($p = 0.9355$), FEV_1/FVC ratio ($p = 0.6856$), PC_{20} histamine ($p = 0.4543$) and bronchodilator reversibility ($p = 0.1609$). All asthmatic subjects were using only intermittent short acting β2-agonist therapy for management of their asthma.

Mucosal expression of pro-inflammatory cytokines (Figures 1 and 2)

The median numbers of bronchial submucosal cells expressing immunoreactive IL-6, IL-8 and IL-17A were significantly elevated in the asthmatic smokers compared to the asthmatic non-smokers ($p = 0.03$, $p = 0.0008$, $p = 0.04$ respectively).

Mucosal infiltration with neutrophils and eosinophils (Figures 1 and 2)

The median number of neutrophils in the submucosa of the airways of the asthmatic smokers was significantly elevated compared with the asthmatic non-smokers ($p =$

0.019). In contrast there was only a trend for an increase in the median numbers of eosinophils in the mucosal sections from the asthmatic smokers compared to asthmatic non-smokers ($p = 0.07$).

Correlations between pro-inflammatory cytokine expression and cellular inflammatory profile (Table 2)

Neutrophil numbers correlated strongly with IL-17A$^+$ cell numbers in the bronchial mucosa of both asthmatic non-smokers and smokers ($r = 0.857$, $p = 0.002$ and $r = 0.907$, $p = 0.002$ respectively). The linear regression model accounted for 74% (non smoking asthmatics) and 82% (smoking asthmatics) of the variance in the number of neutrophils attributed to the number of IL-17A$^+$ cells. In addition, IL-17A$^+$ cell numbers correlated with IL-8$^+$ cell numbers in both asthmatic non-smokers and smokers ($r = 0.636$, $p = 0.048$ and $r = 0.850$, $p = 0.008$ respectively). The numbers of IL-8$^+$ cells correlated with the numbers of neutrophils in the asthmatic non-smokers but not the asthmatic smokers ($r = 0.735$, $p = 0.02$ and $r = 0.678$, $p = 0.06$ respectively).

There were in addition strong correlations between the numbers of eosinophils and the numbers of IL-8$^+$ and IL-17A$^+$ cells in the asthmatic smokers ($r = 0.800$, $p = 0.02$ and $r = 0.863$, $p = 0.006$ respectively), with the linear regression model accounting for 64% and 74% respectively of the variance in the data. These correlations were not evident in the asthmatic non-smokers.

Cigarette smoke extract does not induce ex-vivo expression of IL-17A and IL-17F by respiratory epithelial cells (Figure 3)

The spontaneous expression of both IL-17A and IL-17F by HTEpC was low (3 to 5pg/ml) and not altered in the presence of a concentration series of CSE .

Cigarette smoke extract synergistically increases IL-17A-induced ex-vivo expression of IL-6 and IL-8 by human airways epithelial cells (Figure 4)

IL-17A alone effected a concentration-dependent increase in spontaneous release of IL-6 and IL-8 by HTEpC (ANOVA $p < 0.0001$ and $p < 0.0001$ respectively). The additional presence of CSE further augmented both IL-6 and IL-8 production by these cells in a concentration related, synergistic manner (ANOVA $p < 0.0001$ and $p = 0.008$ respectively).

Aeroallergens increase the expression of IL-6 and IL-8 induced by co-stimulation of epithelial cells with cigarette smoke extract and interleukin-17A (Figure 5)

Spontaneous secretion of IL-6 by cultured airways epithelial cells *ex vivo* was significantly elevated in the presence of 0.16% CSE, 10 ng/ml IL-17A and commercially available cat dander and Timothy grass pollen aeroallergen extracts ($p = 0.009$, 0.01, 0.006 and 0.004 respectively). The combination of CSE and IL-17A increased IL-6 production to a significantly greater degree than either agent alone. While neither aeroallergen significantly altered the effects of CSE or IL-17A alone (cat $p = 0.06$, 0.17; grass $p = 0.27$, 0.98 respectively), both further significantly increased IL-6 production by the combination ($p = 0.0004$, 0.008 respectively). Similarly, spontaneous secretion of IL-8 by the epithelial cells was significantly elevated in the presence of 0.16% CSE, 10 ng/ml IL-17A and the cat dander and Timothy grass pollen extracts ($p = < 0.0001$, 0.0002, 0.01 and 0.01 respectively).

Again, the combination of CSE and IL-17A increased IL-8 production to a significantly greater degree than either agent alone. Again, while neither aeroallergen significantly altered the effects of CSE or IL-17A alone (cat $p = 0.9648, 0.2791$; grass $p = 0.1573, 0.1861$ respectively), both further significantly increased IL-8 production by the combination ($p = 0.03, 0.01$ respectively).

DISCUSSION

Here we report a number of novel observations pertaining to understanding the impact of cigarette smoking and other environmental influences on asthma. Our data are clearly compatible with the hypothesis that exposure of asthmatic patients to environmental cigarette smoke increases *de novo* airways mucosal expression of IL-17A, which in turn acts on structural cells of the mucosa, in this case epithelial cells, to induce the release of granulocyte chemoattractants with resultant influx of neutrophils. Furthermore, we show that cigarette smoke may interact with environmental allergens in a non-antigen-specific (innate) manner to enhance this effect.

Our data are consistent with those of a previous study [26] in which the authors reported elevated IL-17A expression in the bronchial mucosa of mild/moderate asthmatics to a degree which correlated with the numbers of neutrophils in induced sputum, although surprisingly not in the bronchial mucosa. The findings of our *in vitro* experiments are also consistent with a previous study [24] showing that IL-17A induced expression of IL-6 and CXCL8 in primary human airways epithelial cells. We have here extended these findings by demonstrating that CSE is able to augment the expression of both IL-6 and CXCL8 induced by IL-17A in a synergistic manner, implying that cigarette smoke exposure supports and augments IL-17A mediated inflammation of the airways. In a similar vein, Wiehler and Proud [27] have shown synergy between IL-17A and human rhinoviral infection of human airways epithelial cells in inducing the production of CXCL8. Our new data raise the intriguing possibility that inhaled aeroallergens may also interact with cigarette smoke at the airways epithelial barrier in a manner almost certainly independent of acquired

immunity and IgE production, further to augment the expression of IL-6 and CXCL8 in the presence of IL-17A and CSE. We believe that this is the first time that synergistic interaction between IL-17A, cigarette smoke and allergen exposure to induce the expression of pro-inflammatory cytokines has been demonstrated in human airways epithelial cells. It is possible to speculate that differing combinations of environmental influences in different patients conspire to destabilise asthma control through such mechanisms.

While in our study both neutrophilia and CXCL8 expression correlated with IL-17A expression in the bronchial mucosa of both the smoking and the non-smoking asthmatic patients, supporting the hypothesis that IL-17A drives CXCL8 expression in both smokers and non-smokers *in vivo*, CXCL8 expression correlated with neutrophilia only in the non-smoking asthmatic patients, suggesting that in smoking asthmatics IL-17A may induce additional granulocyte chemoattractants. These may include eosinophil, as well as neutrophil chemoattractants given that local IL-17A expression also correlated with eosinophil infiltration in the asthmatic smokers. Possible cellular sources of these chemoattractants other than bronchial epithelial cells include endothelial cells, fibroblasts and smooth muscle cells [10-12]. We did not attempt to identify the IL-17A immunoreactive cells in the asthmatic bronchial biopsies: although contemporary studies implicate Th17 T cells as a likely source, it should be noted that other leukocytes, including γδ and invariant NK T cells, innate lymphoid cells, mast cells and neutrophils are possible alternative sources.

There is considerable circumstantial evidence that IL-17A expression and neutrophilic infiltration of the airways are implicated in the pathogenesis of "difficult to control" asthma, either spontaneous [8, 14] or as a result of smoking [15]. This evidence will

remain circumstantial until mechanisms are uncovered whereby products of granulocytes regulate bronchial hyperresponsiveness, the core pathophysiological abnormality in asthma governing disease severity and stability. Chronic obstructive pulmonary disease (COPD), characterised by severe, irreversible airways obstruction and caused predominantly by cigarette smoking, is also characterised by neutrophil infiltration and elevated IL-17A expression in the airways [26, 28]. In addition to any direct effects that neutrophil products may exert on bronchial hyperresponsiveness in asthma, animal surrogates of allergic inflammation also suggest that neutrophils may play a key role in the development of Th2 type inflammation in atopic dermatitis [29] and asthma [30].

Our study has limitations. Since data on the effects of smoking on asthmatic airways are so limited, we were obliged to make some arbitrary decisions when choosing and classifying the subjects. We arbitrarily defined a smoker as someone who currently smokes ≥ 5 cigarettes per week and a non-smoker as someone not having smoked for at least 12 months prior to screening and with a < 0.5 pack year history. We deliberately avoided setting a minimum pack year smoking history as an exposure criterion for inclusion in the smoking group as this might have admitted chronic, heavy smokers vulnerable to COPD. Similarly, because of the ongoing debate about the possible influence of corticosteroid anti-asthma medication on the numbers of neutrophils in the airways [20] we elected to study mild, corticosteroid naive asthmatics. Clearly, in similar studies, smoking history, disease severity and concomitant corticosteroid therapy of asthmatics will likely modify outcomes [31, 32]. Another intrinsic limitation of the present study is the lack of a non-asthmatic, smoking control group, without which it is impossible to determine whether or not the

smoking-induced changes we observed in the bronchial mucosa of our asthmatic patients were attributable to smoking *per se* or a possible interaction between smoking and asthma. Previous studies on smokers with or without COPD have, however, clearly demonstrated that smoking *per se* fails to induce IL-17A expression in the bronchial mucosa of non-diseased control subjects [26, 28, 33] and that this finding is also reflected in peripheral blood T cells, strongly suggesting that these effects of smoking do indeed reflect an interaction between smoking and asthma [34]. Similarly, the properties of airways epithelial cells may, at least in theory, differ in asthmatics and non-asthmatics, and smoking and non-smoking donors. When the present study was performed, human tracheal, but not bronchial epithelial cells were obtainable commercially (from pooled healthy donors), so we used these as a surrogate. While similarities in the properties of these cells cannot be assumed with certainty, we are aware of no published evidence to the contrary. Finally, we have not addressed the mechanisms by which CSE, IL-17A and allergens influence the function of airways epithelial cells. Montalbano and colleagues have recently demonstrated [35] that both CSE and IL-17A independently increase the production of reactive oxygen species (ROS) by bronchial epithelial cells. In a similar vein, exposure of human bronchial epithelial cells to a variety of pollen aeroallergens has also been shown to increase the production of ROS [25]. We therefore speculate that one mechanism underlying these interacting effects on airways epithelial cells involves the generation of ROS.

In summary, our data suggest that IL-17A is at least one key mediator driving the neutrophilic inflammation seen in asthmatic smokers, and that structural cells of the airways likely play an important role in its pathogenesis. Our data also highlight the

functional significance of the airways epithelium in integrating a variety of environmental influences which may cause such inflammation and influence asthma control.

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CONTRIBUTORSHIP STATEMENT

Dr Leonard Quok Chean Siew had obtained the endobronchial biopsies, performed all the in-vitro experiments and prepared the manuscript. Ms Shih-Ying Wu performed all the immunohistochemistry experiments and reviewed the manuscript. Dr Sun Ying and Professor Christopher John Corrigan was involved in the planning of the study and the in-vitro experiments, as well as the review and editing of the manuscript.

CONFLICTS OF INTEREST

The authors wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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FIGURE LEGENDS

Figure 1: Endobronchial Biopsy immunohistochemistry fast red staining

Endobronchial biopsy sections were stained for IL-6, IL-8, IL-17A, NE and BMK using monoclonal/polyclonal antibodies and fast red staining. Immunoreactive cells are stained red.

Figure 2: Numbers of IL-6, IL-8 and IL-17A immunoreactive cells and elastase+ neutrophils and MBP+ eosinophils in sections of bronchial biopsies

● = Asthma Non-smoker; ○ = Asthma Ex-smoker; ■ = Asthma Smoker

AN = Asthma Non-smoker and AS = Asthma Smoker.

* $p < 0.05$, *** $p < 0.001$, n.s. = not significant.

Figure 3: Cigarette smoke extract does not induce ex-vivo expression of IL-17A and IL-17F by respiratory epithelial cells

HTEpC were cultured with a concentration series of CSE. Spontaneous release of IL-17A (a) and IL-17F (b) was low and not altered in the presence of CSE following 24 hours (open bars) and 72 hours (filled bars) of culture. Mean, n = 2.

Figure 4: Co-stimulation of HTEpC human respiratory epithelial cells with IL-17A and cigarette smoke extract

570 HTEpC were cultured with a concentration series of IL-17A in the presence/absence
571 of CSE. a) and c) CSE synergistically increased IL-17A-induced IL-6 and IL-8
572 secretion (ANOVA $p < 0.0001$ and $p = 0.0079$ respectively). b) and d)
573 Concentration-dependent effect of IL-17A on IL-6 and IL-8 secretion in the presence
574 of CSE (ANOVA $p < 0.0001$ and $p = 0.0079$ respectively). Dotted lines depict the
575 mean increases in baseline expression of IL-6 and IL-8 following stimulation with
576 CSE alone. Paired t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Mean ± SD, $n = 5 –$
577 8.

578 **Figure 5: Effect of cat dander and timothy grass pollen on co-stimulation of**
579 **HTEpC with IL-17A and cigarette smoke extract**

580 Data are presented as the mean ± SD of 4 experiments. Paired t-test. * $p < 0.05$, ** p
581 < 0.01, *** $p < 0.001$, **** $p < 0.0001$.
582

583 **Table 1: Characteristics of the populations studied**

Characteristics	Asthma Non smoker	Asthma Smoker
No.	10	8
Age, yr	27 (24 – 32)	22 (18 – 36)
Height, m	1.77 (1.64 – 1.84)	1.79 (1.60 – 1.92)
Weight, kg	72.6 (66.5 – 81.8)	87.3 (68.9 – 101.5)
Male/Female	6/4	6/2
Never smoker / ex-smoker / current smoker	8/2/0	0/0/8
Pack-years *	0.125, 0.150	4.7 (0.9 – 18.0)
Atopy, No. (%)	10/10 (100)	8/8 (100)
PC_{20} histamine, mg/ml	0.809 (0.440 – 2.01)	1.021 (0.744 – 2.89)
FEV_1, % predicted	93.7 (80.7 – 107.9)	91.4 (78.3 – 112.5)
Pre-bronchodilator FEV_1/FVC, %	77.6 (65.9 – 83.1)	79.3 (69.9 – 84.3)
Bronchodilator response, %	7.5 (3.4 – 14.9)	9.5 (9.0 – 18.9)
St. George's Respiratory Questionnaire Score	13.48 (4.47 – 19.54)	15.59 (9.29 – 21.03)

584 *† $p < 0.05$ compared with asthma non smoker. Mann-Whitney test, Median and IQR.*

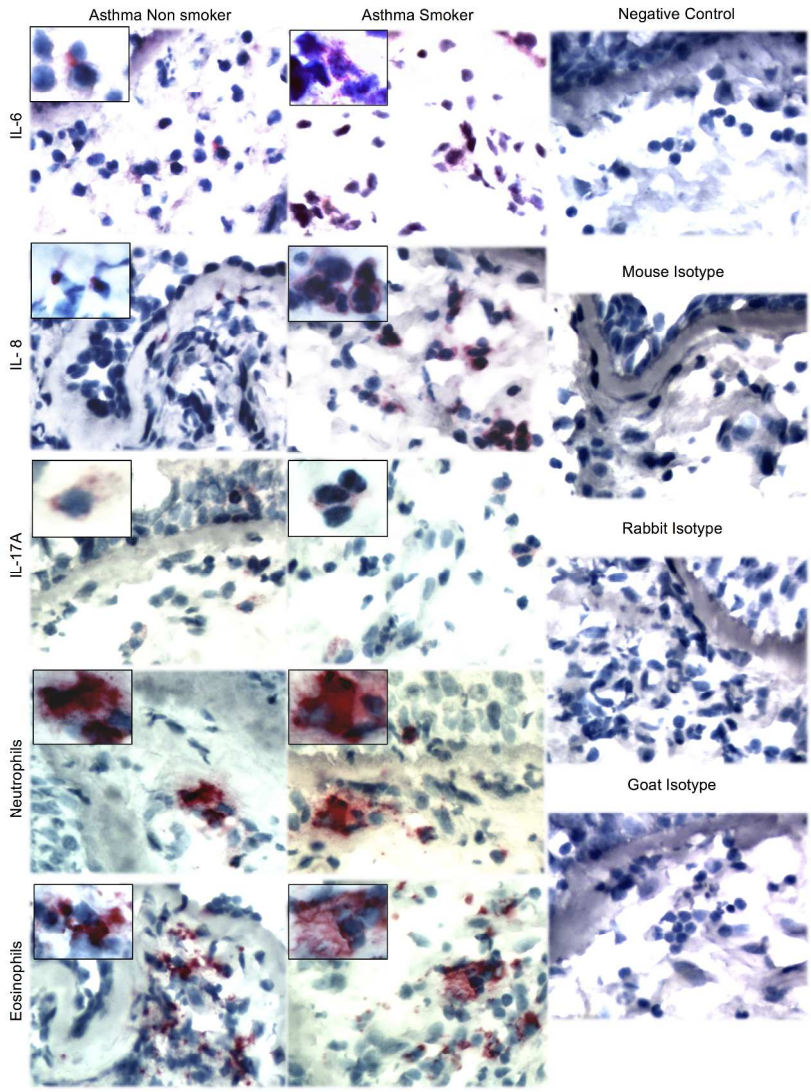
585 ** Two subjects in the asthma non-smoker groups were ex-smokers; pack year*

586 *histories for these subjects are presented individually.*

Table 2: Correlations between pro-inflammatory cytokine expression and cellular inflammatory profile in sections of bronchial biopsies from asthma non-smokers and asthma smokers

	Asthma non-smoker			Asthma smoker		
Correlation	NE+	MBP+	IL-17A+	NE+	MBP+	IL-17A+
IL-8+	**0.735†**	0.564	**0.636†**	0.678	**0.800†**	**0.850§**
	(0.541)	(0.318)	**(0.405)**	(0.460)	**(0.639)**	**(0.722)**
IL-17A+	**0.857§**	0.373	…	**0.907§**	**0.863§**	…
	(0.735)	(0.139)		**(0.822)**	**(0.744)**	

Pearson's r values, r^2 values in parenthesis, significant correlations in bold type. † $p < 0.05$; § $p < 0.01$.



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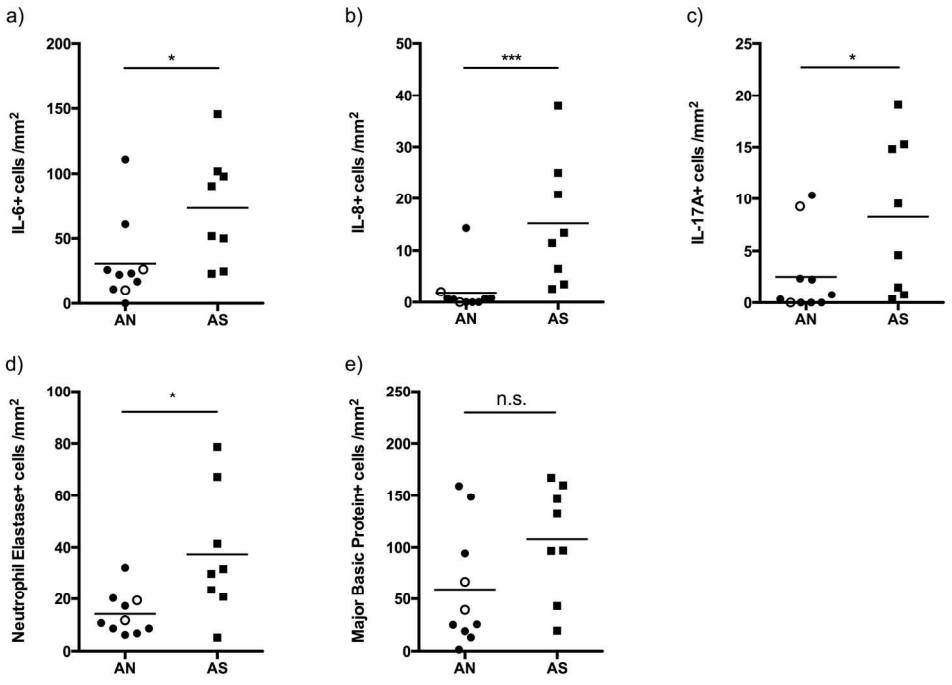
297x420mm (300 x 300 DPI)



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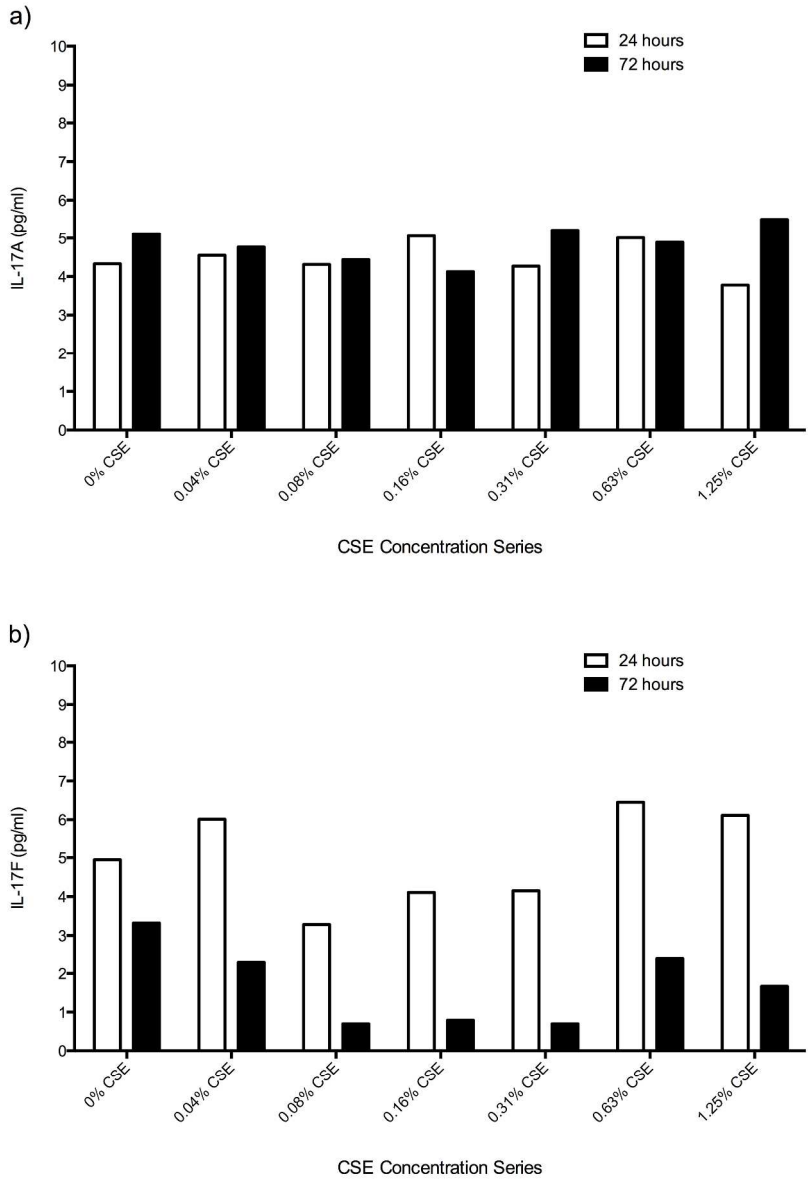
AN = Asthma Non-smoker and AS = Asthma Smoker.
* $p < 0.05$, *** $p < 0.001$, n.s. = not significant.
Figure 2
170x118mm (300 x 300 DPI)

For Peer Review



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240x350mm (300 x 300 DPI)

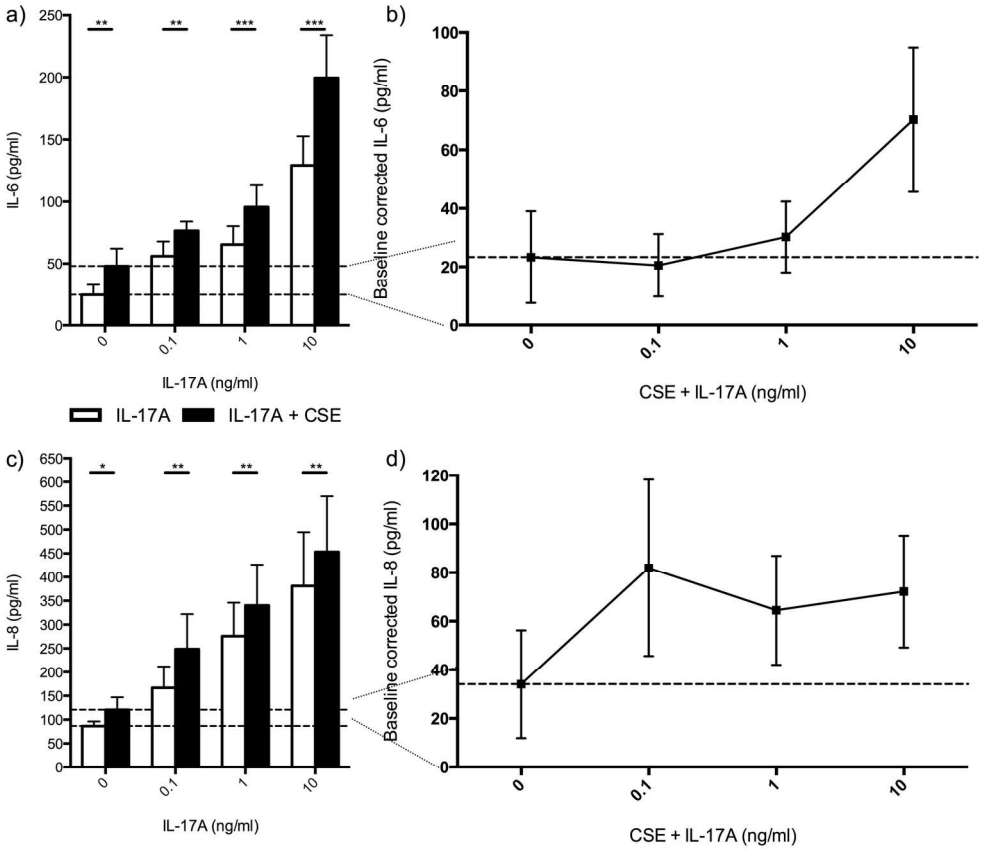

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Figure 4
167x146mm (300 x 300 DPI)

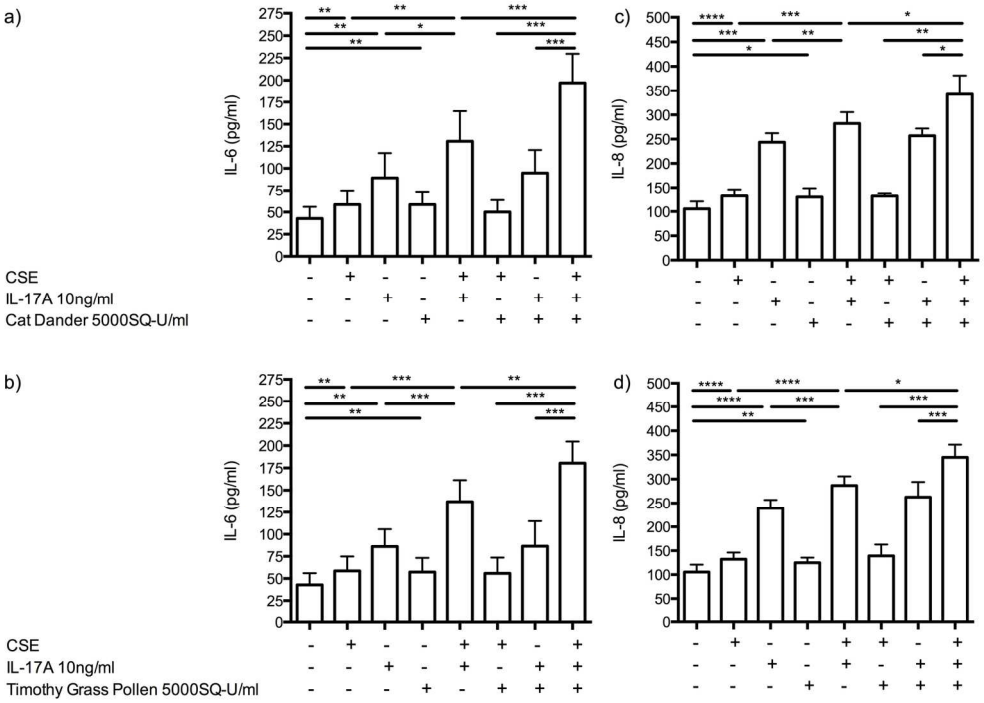

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Figure 5
161x118mm (300 x 300 DPI)

For Peer Review